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Wnt9a Is Required for the Aortic Amplification of Nascent Hematopoietic Stem Cells

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Summary

All mature blood cell types in the adult animal arise from hematopoietic stem and progenitor cells (HSPCs). However, the developmental cues regulating HSPC ontogeny are incompletely understood. In particular, the details surrounding a requirement for Wnt/β-catenin signaling in the development of mature HSPCs are controversial and difficult to consolidate. Using zebrafish, we demonstrate that Wnt signaling is required to direct an amplification of HSPCs in the aorta. Wnt9a is specifically required for this process and cannot be replaced by Wnt9b or Wnt3a. This proliferative event occurs independently of initial HSPC fate specification, and the Wnt9a input is required prior to aorta formation. HSPC arterial amplification occurs prior to seeding of secondary hematopoietic tissues and proceeds, in part, through the cell cycle regulator *myca (c-myc)*. Our

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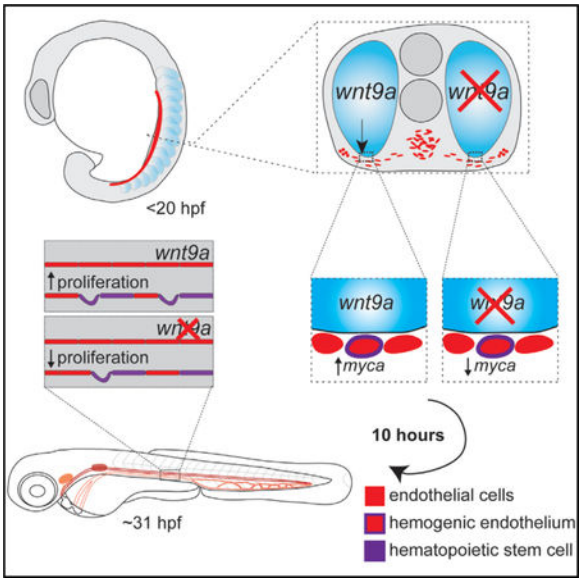
AUTHOR CONTRIBUTIONS

S.G. and J.R. conceived and conducted experiments and performed experimental analysis. S.G. and J.R. wrote the manuscript and made the figures. R.E.P., C.P., B.L., and S.W. performed experiments. K.S.G., W.H., M.R.S., and B.M.W. provided transgenic fish, probes, and reagents. K.W. and D.T. supervised experiments and edited the manuscript.

results support a general paradigm, in which early signaling events, including Wnt, direct later HSPC developmental processes.

Graphical Abstract



In Brief

Hematopoietic stem and progenitor cells (HSPCs) give rise to all of the blood cells of the adult organism; however, how these cells are derived in vivo is still incompletely understood. Using zebrafish, Grainger et al. find that Wnt9a mediates amplification of HSPCs prior to their migration to secondary hematopoietic sites.

INTRODUCTION

Hematopoietic stem cells (HSCs) both self-renew and generate all mature blood cell types throughout the lifespan of the vertebrate organism. Derivation of HSCs in vitro would allow patient-specific replacement therapies. Currently, deriving an HSC in vitro that is suitable for therapeutic use is not possible. Gaining a more thorough understanding of the molecular cues that direct HSC development in vivo will aid in improving protocols to derive HSCs from pluripotent precursors.

HSCs originate from arterial hemogenic endothelium (HE) during vertebrate development, a cell population derived from posterior lateral mesoderm (PLM) in lower vertebrates, which also forms the vascular cord and subsequently the aorta (Brown et al., 2000; Fouquet et al., 1997; Herbert et al., 2009; Jin et al., 2005; Liao et al., 1997). During the migration of PLM cells, inductive cues from the somites are thought to instruct the fate of HE from shared vascular precursors (Bertrand et al., 2010a; Burns et al., 2005; Butko et al., 2015; Clements et al., 2011; Clements and Traver, 2013; Kobayashi et al., 2014; Leung et al., 2013; Wilkinson et al., 2009; Zhen et al., 2013). Hematopoietic stem and progenitor cells (HSPCs) emerge directly from the floor of the dorsal aorta (DA; aorta hereafter) in a process termed

the endothelial-to-hematopoietic transition (EHT) (Bertrand et al., 2010a; Kissa and Herbomel, 2010). In zebrafish, EHT begins at 26 hr post-fertilization (hpf), and the number of emerging HSPCs peaks at around 36 hpf (Bertrand et al., 2010a; Kissa and Herbo-mel, 2010). Following this transition, HSPCs enter circulation through the posterior cardinal vein and migrate to the caudal hematopoietic tissue (CHT) in zebrafish (analogous to the placenta/ fetal liver in mammals) for secondary amplification (Murayama et al., 2006; Tamplin et al., 2015), and finally to the kidney (akin to the mammalian bone marrow), where they reside for the remainder of adult life (Jagannathan-Bogdan and Zon, 2013). Although the anatomical location of these sites varies among organisms, HSC fate is likely instructed by conserved developmental cues. Understanding the inductive signals that instruct HSC fate from the mesoderm will be essential to the eventual derivation of HSCs from pluripotent precursors.

Wnt signaling is vital for the maintenance and development of stem cell populations in many organ systems, including the intestine, skin, and liver (Clevers and Nusse, 2012). *Wnt* genes encode lipid-modified, secreted growth factors that initiate signaling cascades, including the Wnt/β-catenin pathway (commonly referred to as the ‘‘canonical’’ Wnt pathway). Upon Wnt binding its cognate receptor encoded by a *Frizzled* (*Fzd*) gene, the β-catenin protein becomes stabilized and enters the nucleus, where it interacts with the lymphoid enhancer binding factor/T cell factor (LEF/TCF) transcription factors to drive expression of Wnt target genes and regulate a variety of developmental processes.

The role of Wnt function in HSPC development and function remains poorly understood, in part because of conflicting reports. For example, loss of Wnt function depleted the HSPC pool (Fleming et al., 2008; Goessling et al., 2009; Luis et al., 2009; Zhao et al., 2007), and activation of Wnt signaling resulted in an increased HSPC number (Baba et al., 2005; Goessling et al., 2009; Malhotra et al., 2008; Reya et al., 2003; Willert et al., 2003). In contrast, others have observed a depletion of the progenitor pool upon β-catenin overexpression (Kirstetter et al., 2006; Scheller et al., 2006), which may relate to dosage-dependent effects of Wnt signaling on different populations of blood cells (Luis et al., 2011). Although these studies establish that Wnt is essential for the function of adult HSPCs, what is lacking is a clear understanding of Wnt function during the embryonic development of HSPCs. In particular, it is unclear at what stage(s) of HSPC development Wnt is critical. Furthermore, the identification of a specific Wnt ligand regulating HSPC development remains elusive. Here, we demonstrate that Wnt signaling is required prior to formation of the aorta. We further show that the *wnt* gene *wnt9a* is expressed in relevant spatiotemporal do-mains and that HSPCs are depleted following loss of function of *wnt9a;* this loss of function cannot be rescued with ectopic expression of other *wnt* genes. This Wnt9a cue drives an early aortic amplification of HSPCs, which occurs after HSPC emergence begins. This proliferative event is mediated, at least in part, through regulation of *myc-a* (also known as *c-myc*).

RESULTS

Hematopoietic Stem Cell Numbers Are Affected by Wnt Signaling

Using zebrafish, in which complex developmental processes can be readily observed and dissected, we examined the requirement for Wnt signaling during HSPC development. We observed robust Wnt reporter activity in the floor of the dorsal aorta at 26 hpf (during HSPC emergence) using double transgenic *7X TCF:eGFP* (Moro et al., 2012); *kdrl:mCherry* (Bertrand et al., 2010a) embryos, which express eGFP from a Wnt responsive sequence and membrane-bound mCherry in the vasculature (Figure S1A), indicating that endothelial cells have received a Wnt cue.

To monitor the effect of Wnt/β-catenin modulation on HSPCs, we used LiCl, which activates Wnt/β-catenin signaling through inhibition of GSK3b, and IWP-L6 (Wang et al., 2013), which inhibits Porcn, an essential regulator of Wnt ligand maturation and secretion (Kadowaki et al., 1996; Komekado et al., 2007). As previously established, dosages of 0.15 M LiCl or 1.5 mM IWP-L6 did not alter overall embryonic morphogenesis or vasculature, as visualized by *kdrl:mCherry* expression (Figure S1B), but were able to activate or inhibit Wnt signaling, respectively (van de Water et al., 2001; Wang et al., 2013), as measured by expression of the Wnt target gene *axin2* (Jho et al., 2002) (Figure S1C).

HSPCs can be identified as *kdrl:mCherry*; *cmyb:eGFP* double positive cells in the floor of the aorta (Bertrand et al., 2010a). To determine if there was an overall function for Wnt leading to HSPC emergence, we treated larvae from 10 hpf to 36 hpf to activate [LiCl] or inhibit [IWP] Wnt and observed emerging HSPCs at 36 hpf, when their numbers peak (Bertrand et al., 2010a; Kissa and Herbomel, 2010). By doing so, we observed a 2-fold decrease and a 1.5-fold increase in HSPC number after Wnt inhibition [IWP] or activation [LiCl], respectively (Figures 1A and 1B). These effects were confirmed with reverse transcription qPCR for the hematopoietic marker *cmyb* (Figure S1D), indicating that Wnt signaling regulates HSPC number.

Wnt Is Required Transiently Prior to 20 hpf

To identify the window in HSPC development when Wnt is required, we used *hsp:dntcf* transgenic animals, which carry a dominant-negative version of *tcf* (*dntcf*), the expression of which rapidly inhibits expression of Wnt target genes (Figures S1E and S1F) (Clevers and Nusse, 2012; Lewis et al., 2004). We per-formed heat shocks each hour, spanning the window prior to HSPC emergence (13–26 hpf), followed by analysis of *cmyb* expression at 40 hpf by whole-mount in situ hybridization (WISH) (Kissa et al., 2008). Heat shock before 19 hpf resulted in a profound loss of *cmyb* expression in the aorta at 40 hpf, whereas heat shock at 20 hpf or later had no effect (Figures 1C–1E). Because the *dntcf* effect on *axin2* expression occurs acutely and is long-lasting (Figure S1F), these results suggested that the role for Wnt in HSPC development occurs prior to 20 hpf. We confirmed these results with drug treatments (Figure S1G). Specification, when HSCs acquire identity cues, occurs as mesodermal cells migrate to the midline underneath the somites to form the aorta and vein (Kobayashi et al., 2014) (Figure 1I), and can be monitored with early expression of HSPC markers, such as *runx1*. The expression of *runx1* at 26 hpf was unaffected following the drug

treatment regime (Figure S1H) (Burns et al., 2005); *dntcf* expression at 13 hpf also did not affect *runx1* or *cmyb* expression at 29 hpf (Figures S1I and S1J). These results indicate that Wnt signaling positively regulates the number of emerging HSPCs after specification.

We then sought to establish the timeline for the loss of HSPCs in the aorta by inducing expression of *dntcf* at 16.5 hpf and per-forming WISH for *cmyb* from 23 to 36 hpf. Using this approach, we determined that the earliest loss of *cmyb+* cells was detected around 30 to 31 hpf, with an exaggeration of this effect seen through 33 hpf (Figures 1F–1H). Importantly, we could not detect an increase in apoptosis in *dntcf* animals at 32 hpf (Figure S2A). Therefore, in the absence of Wnt, HSPC numbers were diminished by a mechanism independent of apoptosis.

Wnt Cue Is Required in Cells of the HE

To test whether the Wnt signal is received by endothelial cells, we generated upstream activating sequence *(UAS):dntcf* and *UAS:constitutively active (CA)-β-catenin* transgenic animals to drive the previously reported *dntcf* or *CA-β-catenin* transgenes with Gal4 in the vasculature (Figures S2B–S2E). Similar to the ubiquitous induction of the *hsp:dntcf* transgene, endothelial-specific (using *fli1a:Gal4*) Wnt inhibition resulted in a loss of HSPCs at 40 hpf (Figures 2A–2C), whereas *CA-β-catenin* expression led to an increase in HSPCs at 40 hpf (Figure S2F), indicating that the Wnt signal acts upon vascular cells to influence HSPC development.

To determine if the Wnt cue is required broadly in endothelial cells, or more specifically in HE, we drove *dntcf* using a *gata2b:Gal4* driver, which is expressed in the earliest known population of HE (the transient population of endothelial precursors that can differentiate into HSCs) (Butko et al., 2015). Similar to ubiquitous *hsp:dntcf* and endothelial-specific transgenes, Wnt inhibition in *gata2b*$^{+}$ cells resulted in a loss of HSPCs at 40 hpf (Figures 2D–2F), indicating that the Wnt signal acts upon HE to influence HSPC development.

Wnt9a Is Required for HSPC Development

The data presented above establish an essential role for Wnt signaling in HSPC development. We have previously demonstrated that the ventral somite signals to neighboring endothelial precursors during HSPC migration to the embryonic midline (Clements et al., 2011; Kobayashi et al., 2014). The timing of the Wnt requirement in HSPC development suggested the somite as a possible source of Wnts. To identify candidate *wnt* gene(s), we surveyed expression of 21 *wnt* genes by qPCR in *myf5-GFP* positive (somitic) cells (Chen et al., 2007) prior to (16.5 hpf) and after (20 hpf). We identified *wnt9a* as our prime candidate because it is expressed at 16.5 hpf, and downregulated at 20 hpf (Figures S3A–S3C). By WISH, *wnt9a* transcript is detected in the posterior somites at 16.5 hpf; in regions consistent with vasculature at 19 hpf; and in the vasculature and the CHT by 28 hpf (Figure 3A).

To determine the effect of loss of *wnt9a* on HSPC development, we used a splice-blocking morpholino (MO) to knock down *wnt9a* expression in embryos (Figures S3D and S3E). Upon injection into embryos, this MO decreased Wnt signaling, as demonstrated by reduction in *axin2* expression (Figure S3F). We observed a 2-fold decrease in *kdrl:mCherry*;

cmyb: eGFP HSPCs emerging from the floor of the dorsal aorta at 36 hpf using this MO and a second MO designed to block translation of *wnt9a* (ATG-MO) (Figures 3B–3D). This phenotype was specific to the hematopoietic system because the vasculature, aorta, and pronephros were properly specified in MO-injected embryos (Figure S3G). MO knockdown of the closely related *wnt9b* gene did not affect HSPCs, suggesting that *wnt9a* is specifically required for HSPC development (Figures 3C and 3D). To further determine whether or not Wnt9a specifically instructs HSPC development, we performed co-injections of cDNA (to circumvent early lethality) encoding either *wnt9a*, *wnt9b*, or *wnt3a* with the *wnt9a* splice-blocking MO. Ectopic expression of *wnt9a* rescued HSPC numbers in *wnt9a* morphants (Figures 3C and 3D). In contrast, ectopic expression of *wnt9b* or *wnt3a* did not rescue the *wnt9a* MO effect on HSPC numbers (Figures 3C and 3D). These data suggest that Wnt9a specifically drives HSPC development, and that loss of *wnt9a* cannot be rescued by overexpression of other *wnt* genes.

The loss of HSPCs in *wnt9a* morphants persisted to later stages of embryonic hematopoiesis: at 4 days post-fertilization, we observed a dose-dependent loss of *cmyb*+ cells in the CHT, indicating a substantial decline in the total number of HSPCs (Figure 3E). Similar to enforced expression of *dntcf*, *wnt9a* knockdown had no effect on *runx1* expression at 26 hpf (Figures S3H and S3I). Finally, because recent reports have indicated that MOs may have non-specific effects (Kok et al., 2015; Schulte-Merker and Stainier, 2014; van Impel et al., 2014), we confirmed that genetic mutation of *wnt9a* results in loss of *cmyb*$^{+}$ cells at 36 hpf as well (Figures 3F–3H). We confirmed that somitic *wnt9a* is required for HSPC emergence by injecting a *UAS:wnt9a* cDNA construct into fish with a somitic gal4 driver (*phldb1:gal4*), in the context of the *wnt9a* MO, and found that somitic overexpression of *wnt9a* was sufficient to partially rescue the loss of HSPCs (Figures S3J and S3K). Taken together, these results indicate a defect in the emergence of HSPCs following loss of *wnt9a*.

Hematopoietic Precursors Expand in the Aorta

Next, we aimed to determine the mechanism underlying HSPC reduction upon loss of Wnt function. HSCs arise from hemogenic cells in the aorta, enter circulation, and seed the CHT, where they proliferate and differentiate before migrating to the adult hematopoietic tissues (Murayama et al., 2006). The observation that diminished Wnt signaling led to a decrease in aortic HSPCs suggested that HSPCs also undergo an expansion in the aorta. HSPCs can also be detected using *kdrl:mCherry; gata2b:GFP* double transgenic animals (Butko et al., 2015). We used this line for EdU pulse labeling at 26 hpf to detect proliferation events in emerging HSPCs at 33 hpf. With this approach, we could detect gata2b+ cells that had proliferated, prior to exit from the aorta (Figures 4A and 4B). We also detected proliferation events in *kdrl:mCherry; cmyb:GFP* animals at 32 hpf and as early as 30 hpf (Figures S4A and S4B). To determine the extent to which HSPCs proliferate in the aorta, we treated *kdrl:mCherry; gata2b:GFP* animals from 26 to 35 hpf with 5-fluorouracil, which selectively kills cells that have undergone DNA synthesis (Heidelberger et al., 1957). This resulted in a 2.5-fold reduction in the average number of HSPCs at 36 hpf (Figures 4C–4E), confirming a requirement for amplification of nascent HSPCs in the aorta.

Wnt9a Drives HSPC Proliferation in the Aorta through myca

The loss of HSPCs independent of apoptosis and the concomitant requirement for proliferation in the aorta suggested that in the absence of a Wnt cue, HSPCs may be G1 arrested; the FUCCI fish identifies G1-phase cells with the red fluorescent protein mCherry (Bouldin and Kimelman, 2014; Sugiyama et al., 2009). Endothelial cells in the G1 phase can therefore be sorted from *kdrl:GFP; FUCCI* morphant fish and compared to the same population from uninjected controls. We sorted G1-phase endothelial cells (*kdrl:GFP; FUCCI*) from morphant and uninjected fish. At 28 hpf, G1 cells from *wnt9a* MO-injected fish had robust expression of *gata2b*, whereas we were unable to detect *gata2b* transcripts in the uninjected sample; *gata2b* expression in un-sorted fish was similar in morphant and control fish, suggesting that HSPCs are G1 arrested in the absence of *wnt9a* (Figures 4F and 4G). We could also detect an increase in G1-arrested endothelial cells in *wnt9a* MO-injected animals (Figures S4C and S4D).

Entrance to the replicative S phase of the cell cycle is governed by a series of cellular events requiring D-class cyclins and their cofactors, cdk2 and cdk4 (Bertoli et al., 2013), which we predicted to be decreased in Wnt-reduced animals. Consistent with this model, we observed a profound loss of *cyclinD2b* and *cdk4,* and a small, but significant, decrease in *cyclinD1* and *cdk2* in Wnt-suppressed (*dntcf*+) endothelial cells at 30 hpf (Figures 5A and B). The transcriptional regulator and contextspecific Wnt target gene *myca*, which acts upstream of these cell cycle regulators (Mateyak et al., 1999), was also downregulated in *dntcf*+ endothelial cells (Figure 5B), whereas normal *myca* levels increase in endothelial cells during development, concordant with endothelial expansion (Figure S5A). We could not detect a loss of these cell cycle regulators at earlier time points, such as 24 hpf (Figure S5B). Accordingly, introducing *myca* mRNA in *wnt9a* morphants was sufficient to rescue the loss of *cmyb*+ cells at 40 hpf (Figures 5C and 5D) and of cell cycle regulators by 28 hpf (Figure S5C), indicating that at least a portion of Wnt9a function in HSPCs proceeds through *myca*. Finally, expressing *myca* expression under control of the *gata2b* promoter (in *gata2b:KalTA4; UAS:myca*) was sufficient to rescue the loss of *wnt9a* in morphants (Figures 5C and 5D), further supporting this requirement in the HE. Taken altogether, our results indicate that Wnt9a drives an early amplification of HSPCs up- stream of the cell cycle regulator *myca* in the HE (Figure 6).

DISCUSSION

Wnt signaling regulates multiple stages of hematopoiesis, but requirements for the specific molecules that mediate these signals are not well understood. This study sought to understand the regulation of specific *wnt* genes that instruct early hematopoietic development. We found that a single *wnt* gene, *wnt9a*, mediates a critical Wnt signal that is received by the endothelium prior to 20 hpf for HSPC emergence in the zebrafish. The Wnt9a signal stimulates a previously unrecognized HSPC amplification event in the aorta that is mediated through activation of *myca,* a Wnt-regulated gene, and important regulator of cell proliferation.

Previous work has demonstrated an early Wnt signaling requirement for the production of long-term HSCs in mouse and zebrafish (Goessling et al., 2009; Ruiz-Herguido et al., 2012);

however, the identity of Wnt signaling components, particularly which of the numerous Wnts, has remained unclear. Here, we demonstrate a unique requirement for the somatically expressed *wnt9a* in HSPC development. Knockdown of *wnt9a* causes a decrease in *cmyb*+ hematopoietic precursors at 36 hpf, consistent with previous global knockdown of Wnt function in mouse and zebrafish (Goessling et al., 2009; Luis et al., 2009, 2011; Ruiz-Herguido et al., 2012). Extending these studies, we have found that loss of *wnt9a* expression does not cause a defect in HSPC specification, as evidenced by normal *runx1* expression in the aorta at 26 hpf. This is also in contrast to other factors known to affect HSPC development at the level of specification (Butko et al., 2015; Clements et al., 2011; Espín-Palazón et al., 2014; Kim et al., 2014; Kobayashi et al., 2014; Lee et al., 2014; Pouget et al., 2014). For example, Wnt16 acts through a non-canonical Wnt pathway upstream of Notch signaling to specify HSPC identity (Clements et al., 2011), a process that is mediated by an interaction between the ventral somite and migrating vascular precursors (Kobayashi et al., 2014).

Loss of *wnt9a* cannot be compensated for by overexpression of other *Wnt genes*, suggesting that the requirement for Wnt9a in HSPC development is specific. This specificity is surprising because in many experimental settings, individual Wnt proteins produce similar effects and are often interchangeable. Our current understanding of specific Wnt interactions with their cognate receptors is quite limited and largely restricted to in vitro studies. For example, wingless (the *Drosophila* Wnt1 ortholog) interacts with the cysteine-rich domains (CRDs) of both Fz and Dfz2 (two *Drosophila Fzd* proteins that act redundantly in establishing segment polarity in the embryo), but with 10-fold lower affinity for Fz than for Dfz2 (Rulifson et al., 2000). Determining specificities of Wnts for their receptors is confounded by the large number of Wnts and Wnt receptors involved: the mammalian genome contains 19 *wnt* and 10 *Fzd* genes and the zebrafish genome contains 20+ *wnt* and 14 *fzd* genes. A recent study analyzed the interactions of four Wnt proteins with six Fzd CRDs and found a significant range in binding affinities among individual pairs (Dijksterhuis et al., 2015). Identifying the Fzd(s) and co-receptors expressed in the pre-HE during HSPC development will be informative in studying the interaction of Wnt9a with these potential receptors.

Wnt9a is well conserved among vertebrates (Curtin et al., 2011; Kamel et al., 2013) and is expressed in mouse HSPCs (Wu et al., 2012), indicating possible conservation of function during hematopoietic development. In this context, it is worth noting that in both zebrafish and mammals, the *wnt9a* gene is syntenic to *wnt3a* (Nusse, 2001), suggesting coordinate regulation of these two *wnt* genes. Importantly, Wnt3a has been implicated in HSC self-renewal in the mouse (Luis et al., 2009; Willert et al., 2003), but does not appear to have a role in zebrafish hematopoiesis (Buckles et al., 2004; Clements et al., 2009; Thorpe et al., 2005). A role for Wnt9a in mammalian hematopoietic development has not yet been addressed.

Interestingly, zebrafish embryos that are deficient for Wnt signaling do not show a hematopoietic phenotype until 30 hpf, which is 10 hr removed from the time that the signal is required. This delayed effect of a Wnt signal may be related to the concept of cellular memory put forth by Vincent and colleagues, who pro-posed that earlier signaling events

allow persistent expression of relevant target genes (Alexandre et al., 2014). Our data indicate that during this time, cells of the HE are primed for amplification in the dorsal aorta. Upon loss of Wnt signaling via global pathway inhibition or *wnt9a* knockdown, these emerging HSPCs are arrested in the G1 phase of the cell cycle, causing a decrease in proliferating HSPCs and overall HSPC numbers. This regulation of the cell cycle is mediated, at least in part, by *myca* (the zebra-fish homolog of mammalian *C-MYC*), which, among other targets, controls transcription of D-class cyclin genes and their associated cyclin-dependent kinases (Amati et al., 1998; Hanson et al., 1994; Mateyak et al., 1999). These genes are downregulated when Wnt is inhibited, and loss of HSPCs in this context can be rescued with *myca*. Our findings are consistent with re-ports showing that MYC expression is vital to maintaining HSPC numbers and function (Delgado and León, 2010; Laurenti et al., 2008) and studies using MYC as a factor to facilitate re-programming to HSPC fate (Riddell et al., 2014), and also that MYC is a context-dependent Wnt target (Cole et al., 2010; Kolligs et al., 1999; Muncan et al., 2006; Sansom et al., 2007). Although it appears that Myca operates downstream of the Wnt9a cue, further investigation will be required to determine the nature of the timing delay between 20 and 31 hpf because both *myca* transcript and protein are known to be tightly controlled, with estimated half-lives of 20–30 min or less (McCormack et al., 1984; Rabbitts et al., 1985). Interestingly, Myca and Notch1 (which is critical to HSC fate specification) have been previously shown to interact (Bertrand et al., 2010b; Bigas et al., 2013; Burns et al., 2005; Kim et al., 2014; Kumano et al., 2003; Palomero et al., 2006). However, we could not detect any differences in Notch reporter expression in *wnt9a* morphants (data not shown), suggesting that this process occurs independently of Notch signaling. This suggests that tight regulation of cell proliferation by the Wnt signaling pathway through myca is critical for proper hematopoietic development.

Our findings show a unique role for Wnt9a in zebrafish HSPC development. This signal is received by cells of the HE as they ingress to the midline to form the vascular cord prior to 20 hpf. The Wnt9a signal instructs HSPC emergence, but not HSPC specification, via priming HSPCs for later aortic amplification. The specific Wnts necessary to differentiate human pluripotent stem cells to hematopoietic or other lineages are often unknown or unused in protocols. Instead, global small molecule pathway activators or inhibitors are often favored because of their wide- spread availability and inexpensive nature. Our data indicate that the specific molecules that mediate a specific signal during in vivo development may provide more precise developmental instruction than small molecules with non-specific effects on signaling pathways, especially because differences in Wnt requirements could also be reflective of precise timing and ligand requirements. Understanding the precise identity of these instructive signals and their temporal regulation is critical for improving differentiation protocols to develop HSCs in vitro, which one day could be used as a therapeutic alternative to bone-marrow transplants.

EXPERIMENTAL PROCEDURES

Zebrafish Lines and Maintenance

Zebrafish were maintained and propagated according to University of California and local institutional animal care and use committee policies (protocol S04168). AB*,

Tg(cmyb:eGFP)^{zf169Tg}, *Tg(hsp:Gal4)*$^{kca4Tg/+}$, *Tg(kdrl:Cherry-CAAX)*^{y171}, *Tg(fli1a:eGFP), Tg(kdrl:eGFP)*^{s843}, *Tg(7X TCF-X.laveis-siamois: eGFP)*^{ia4}, *Tg(fli1a:Gal4), Tg(gata2b:KalTA4; UAS:Lifeact:eGFP), Tg(Dual FUCCI), Tg(fli1a:EcRF-VP16), Tg(phldb1:KalTA4)*, and *Tg(hsp70l:dntcf7l1a)*^{w26} lines have been previously described (Bertrand et al., 2008, 2010a; Bouldin and Kimelman, 2014; Butko et al., 2015; Espín-Palazón et al., 2014; Jin et al., 2005; Kobayashi et al., 2014; Lawson and Weinstein, 2002; Lewis et al., 2004; Moro et al., 2012; North et al., 2007; Sugiyama et al., 2009; Swift et al., 2014). *Tg(UAS-dntcf7l1a-CG2), Tg(UAS: wnt9a)* and *Tg(UAS:myca)* founders were established by injecting 25 pg of the constructs described below with 100 pg of transposase mRNA at the one-cell stage. For simplicity in the text, these lines are referred to with shortforms listed in square brackets: *Tg(cmyb:eGFP)*^{zf169Tg} *[cmyb:eGFP], Tg(kdrl:Cherry-CAAX)*^{y171} *[kdrl:mCherry], Tg(fli1a:eGFP) [fli1a:eGFP], Tg(kdrl:eGFP)*^{s843} *[kdrl:eGFP], Tg(7X TCF-X.laveis-siamois:eGFP)*^{ia4} *[7XTCF:eGFP], Tg(gata2b:KalTA4; UAS:Lifeact:eGFP) [gata2b:eGFP]* and *Tg(hsp70l:dntcf7l1a)*^{w26} *[hsp:dntcf], Tg(UAS-dntcf7l1a-CG2)[UAS:dntcf], Tg(UAS: wnt9a) [UAS: wnt9a], Tg(UAS:myca) [UAS:myca], Tg(phldb1:KalTA4) [phldb:KalTA4]*, and *Tg(Dual FUCCI) [FUCCI].* MOs for *wnt9a* were targeted to retain the first intron (Figure S3D) with sequence 50-GAAAGAATTGTCCTGCCTACCCGAA-3′, or targeted to block the ATG start codon (*wnt9a* ATG-MO) with sequence 50-CCAGGAGAAGGTGTCCATCCAG CAT-3′ from GeneTools. One-cell stage zygotes were injected with 1 ng of *wnt9a* MO, and retention of the intron was confirmed by PCR. The ATG-MO was used at a concentration of 2 ng per injection. Mutation of the *wnt9a* locus was achieved by injecting 100 ng of cas9 mRNA (Trilink) and 100 ng of short guide RNA (sgRNA) targeting exon 1 (ATTGGGACGGCTAATAGATT). Muta-tions were confirmed by sequencing individuals. Modulation of Wnt signaling was carried out using 0.15 M LiCl or 1.5 mM IWP-L6 in E3 with PTU as previ-ously described (van de Water et al., 2001; Wang et al., 2013). For heat shock experiments, fish were heat shocked at 38 C for 10 or 30 min and were allowed to return to 28.5 C gradually. 5-Fluorouracil treatments were performed at 10 mM, with DMSO as a vehicle. Rescue experiments were performed using 10-pg *myca* mRNA synthesized using the SP6 mMessage machine kit (Ambion), according to the manufacturer's recommendations.

WISH

RNA probe synthesis was carried out according to the manufacturer's recommendations using the DIG-RNA labeling kit (Roche). Probes for *dll4, hey2, notch1b*, *msr*, *kdrl, cdh17, cmyb*, and *runx1* and WISH protocols have been previously described (Clements et al., 2011; Kobayashi et al., 2014; Rowlinson and Gering, 2010), whereas the probe construct for *wnt9a* was a gift from W. Herzog.

Fluorescence-Activated Cell Sorting and qPCR

Zebrafish were dissociated using Liberase TM (Roche) and filtered through an 80-μm filter. Cells were sorted on a BD Influx cell sorter according to standard procedures. RNA and cDNA were synthesized by standard means and qPCR was performed using FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturer's

recommendations and analyzed using the 2-ΔΔCt method (Schefe et al., 2006). Sequences of primers are shown in the Supplemental Experimental Procedures.

EdU and TUNEL Labeling

Zebrafish larvae were injected with 1 nL of 10 mM EdU into the heart at 26 hpf, fixed at later time points, and stained using the Click-iT EdU imaging kit (Invitrogen) according to the manufacturer's recommendations. Transgenic reporters were stained using standard immunofluorescence with chicken anti-GFP (Aves Labs) and anti-mCherry [1C51] (abcam). TUNEL staining was carried out as previously described (Espín-Palazón et al., 2014).

Quantifying HSPCs

HSPCs were quantified by counting the number of *kdrl:mCherry; cmyb:eGFP* or *kdrl:mCherry; gata2b:GFP* double positive cells in the floor of the DA in the region above the yolk extension in a 625-μm confocal Z stack encompassing the entire mediolateral segment of the aorta. The number of HSPCs per millimeter was calculated from these data. Confocal images were generated by stacking one to four individual Z slices. When quantifying WISH data, the number of *cmyb*+ cells was counted above the yolk extension.

Plasmid Construction

Zebrafish *dntcf7l1a* lacking the β-catenin-binding domain was amplified by PCR and subcloned to pENTR1a dual selection and recombined with p5E-UAS, p3E-pA, and the Tol2 cmlc2:eGFP destination vector from the Tol2 kit (Kwan et al., 2007). Constructs to express zebrafish *myca*, *wnt3a*, *wnt9a*, and *wnt9b* were established by subcloning cDNA encoding each gene into pCS2+.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- An early Wnt cue directs later HSPC proliferation, prior to secondary amplification
- Wnt9a is uniquely required for early HSPC amplification
- Myc is downstream of the Wnt cue necessary for proliferation

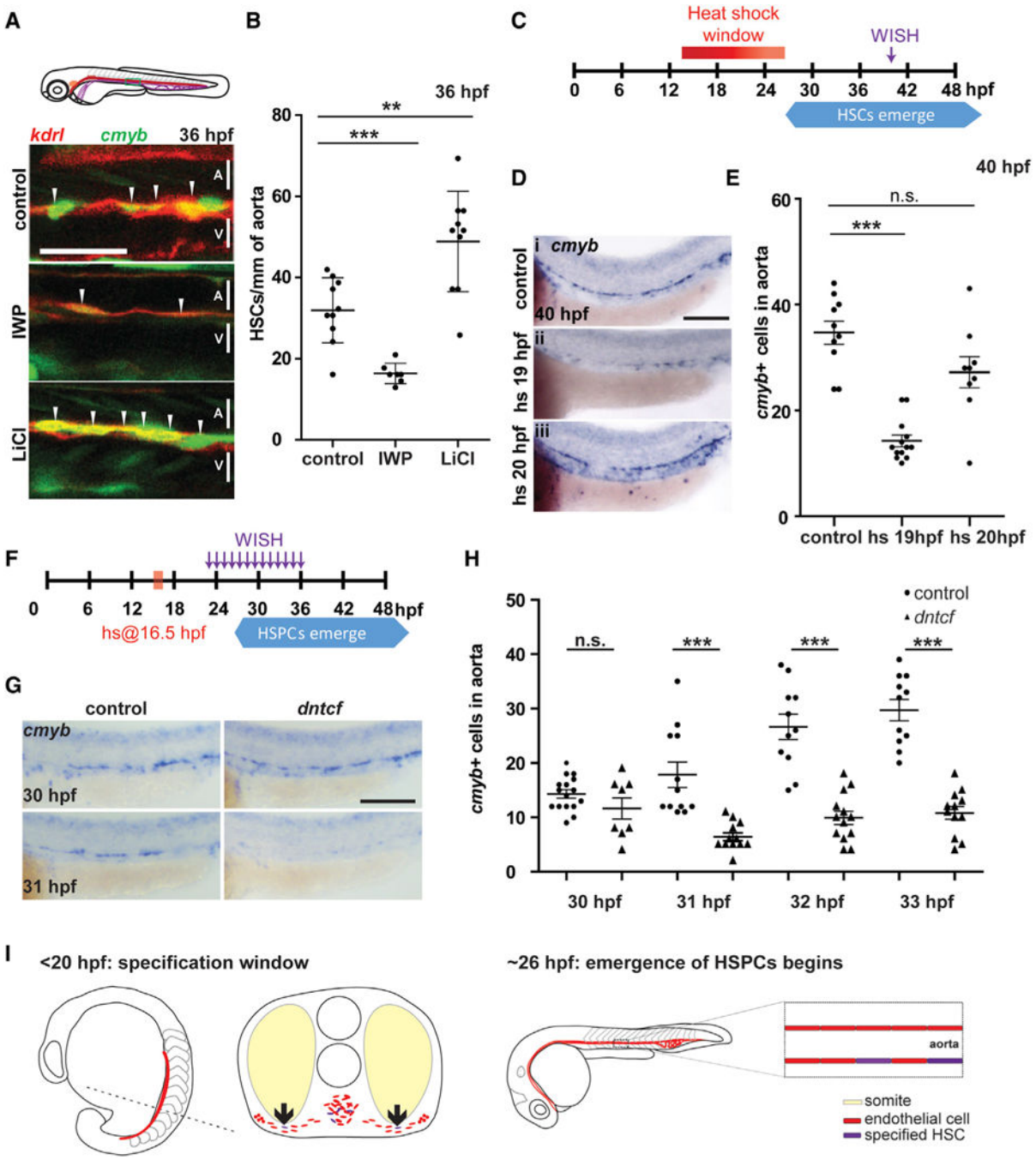

Figure 1. Wnt Signaling Is Required Transiently Prior to 20 hpf for HSPC Development

(A) *kdrl:mCherry*; *cmyb:eGFP* fish were treated with IWP-L6 or LiCl to inhibit and activate Wnt signaling, respectively (van de Water et al., 2001; Wang et al., 2013), from 10 hpf to 36 hpf and imaged at 36 hpf. A, aorta; V, vein. Scale bar, 30 mm.

(B) Quantitation of HSPCs per millimeter of aorta.

(C) Schematic of heat shock regimen.

(D) *hsp:dntcf* fish were heat shocked every hour from 13 hpf to 24 hpf, fixed at 40 hpf, and analyzed for *cmyb* expression by WISH. Scale bar, 100 mm.

(E) Quantitation of *cmyb+* cells from (D).

(F) Schematic of experimental layout.

(G) *hsp:dntcf* fish were heat shocked at 16.5 hpf, pools were fixed every hour from 23 to 36 hpf, and they were analyzed for *cmyb* expression by WISH. Scale bar, 100 μm.

(H) Quantitation of *cmyb*+ cells from (G).

(I) HSPCs are specified as endothelial cells ingress toward the midline. Endothelial cells receive specification cues, at least in part, from somatic cells. At 26 hpf, HSPCs start to emerge from the floor of the dorsal aorta. A, aorta; V, vein.

**p < 0.01; ***p < 0.001; n.s., not significant. Error bars represent SD. See also Figure S1.

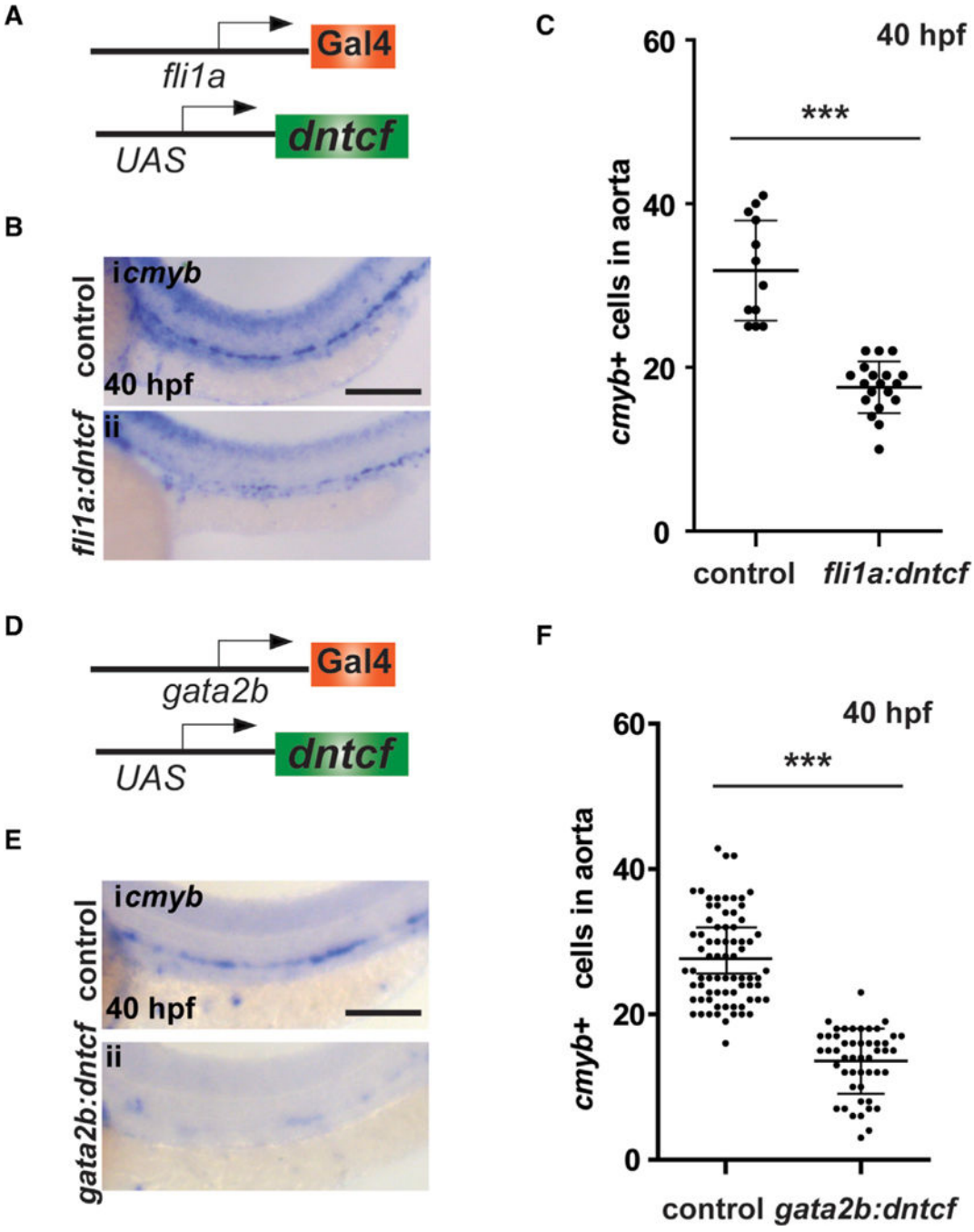

Figure 2. Wnt Cue Is Required in Cells of the HE

(A) Schematic of transgenic elements used for endothelial specific expression of *dntcf* in endothelial cells.

(B) WISH for *cmyb* at 40 hpf in control and in *fli1a:Gal4; UAS:dntcf* fish.

(C) Quantitation of *cmyb*+ cells from (B).

(D) Schematic of transgenic elements used for endothelial specific expression of *dntcf* in the HE.

(E) WISH for *cmyb* at 40 hpf in control and in *gata2b:Gal4; UAS:dntcf* fish.

(F) Quantification of *cmyb*+ cells from (E).

***p < 0.001. Scale bars, 100 μm. Error bars represent SD. See also Figure S2.

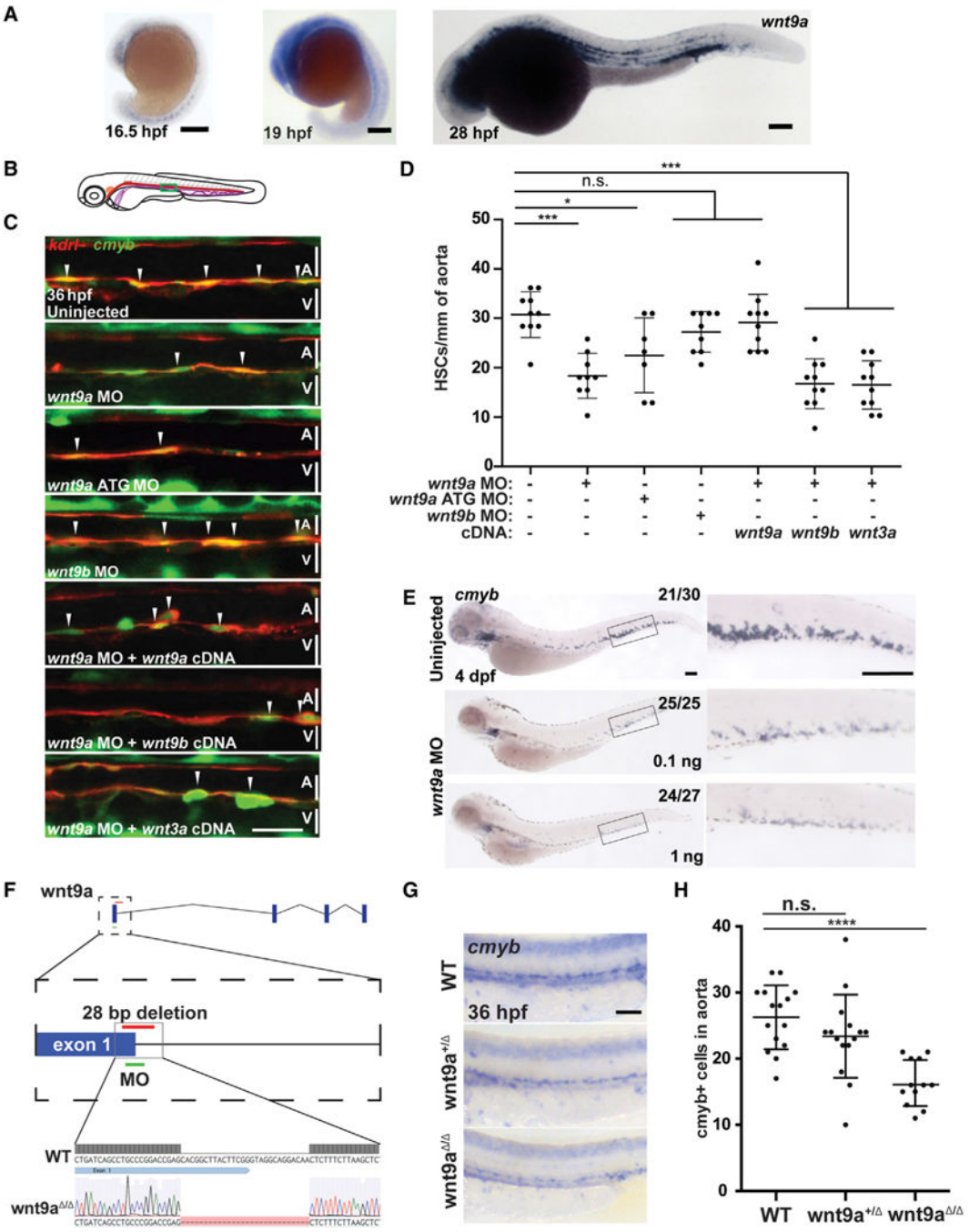

Figure 3. Wnt9a Is Required for HSPC Formation

(A and B) WISH for *wnt9a* at 16.5 hpf, 19 hpf, and 24 hpf (A). Scale bars, 0.2 mm; representative images of n = 15. Imaged area is shown in (B).

(C and D) Representative images of *kdrl:mCherry*; *cmyb:eGFP* injected as listed (C). HSPCs per millimeter of aorta are quantified in (D). Scale bars, 30 mm.

(E) WISH for *cmyb* in 4 days post fertilization (dpf) fish treated with 0.1 ng or 1 ng of *wnt9a* MO. Scale bars, 0.2 mm.

(F) *Wnt9a* mutants were generated by injection of guide RNA targeting the last portion of the first exon. (G and H) Expression of *cmyb* was examined by WISH in 36 hpf WT, *wnt9a* $^{+/D}$ and *wnt9a*$^{D/D}$ zebrafish

(G) and quantified in (H).

Scale bars, 100 mm. *p < 0.05; ***p < 0.001; n.s., not significant. Error bars represent SD. See also Figure S3.

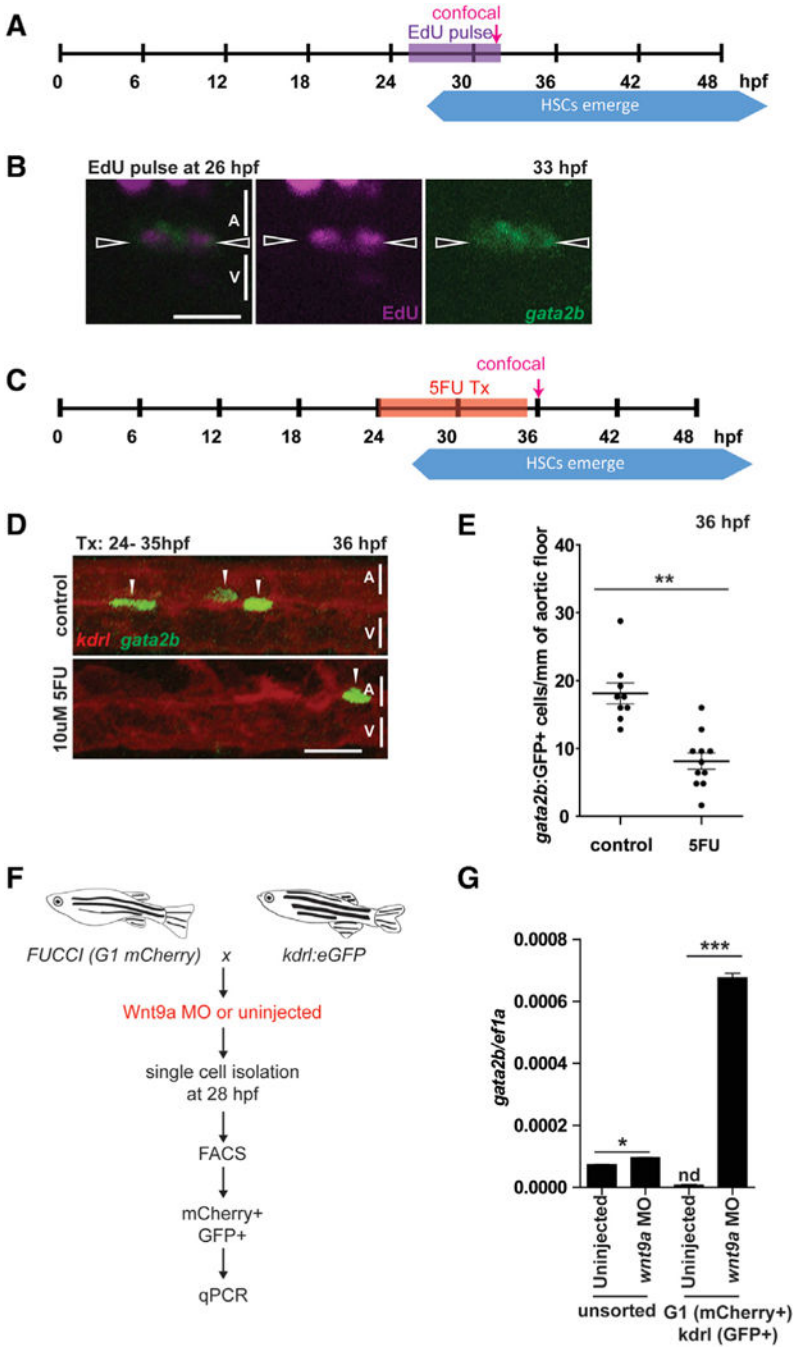

Figure 4. HSPCs Expand in the Aorta

(A) The heart tubes of *gata2b:GFP* fish were injected with 1 nL of 10 mM EdU at 26 hpf, and fixed and stained for cells that had divided as well as GFP at 33 hpf (n = 10).

(B) Representative confocal image of *gata2b*+ cells that have undergone cell division in the aorta (arrowheads).

(C–E). *gata2b:GFP;kdrl:mCherry* fish were treated with 10 mM 5′ fluorouracil (5FU) from 24 to 35 hpf (C), confocal imaged at 36 hpf (D), and *gata2b+* cells quantified (E).

(F and G) G1 arrested (mCherry+) endothelial cells (GFP+) from *wnt9a* mor-phant and control fish (n = 100 embryos per condition) were collected by FACS at 28 hpf (F) and compared by qPCR for *gata2b* (G).

Scale bars, 30 μm. Error bars represent SD. See also Figure S4.

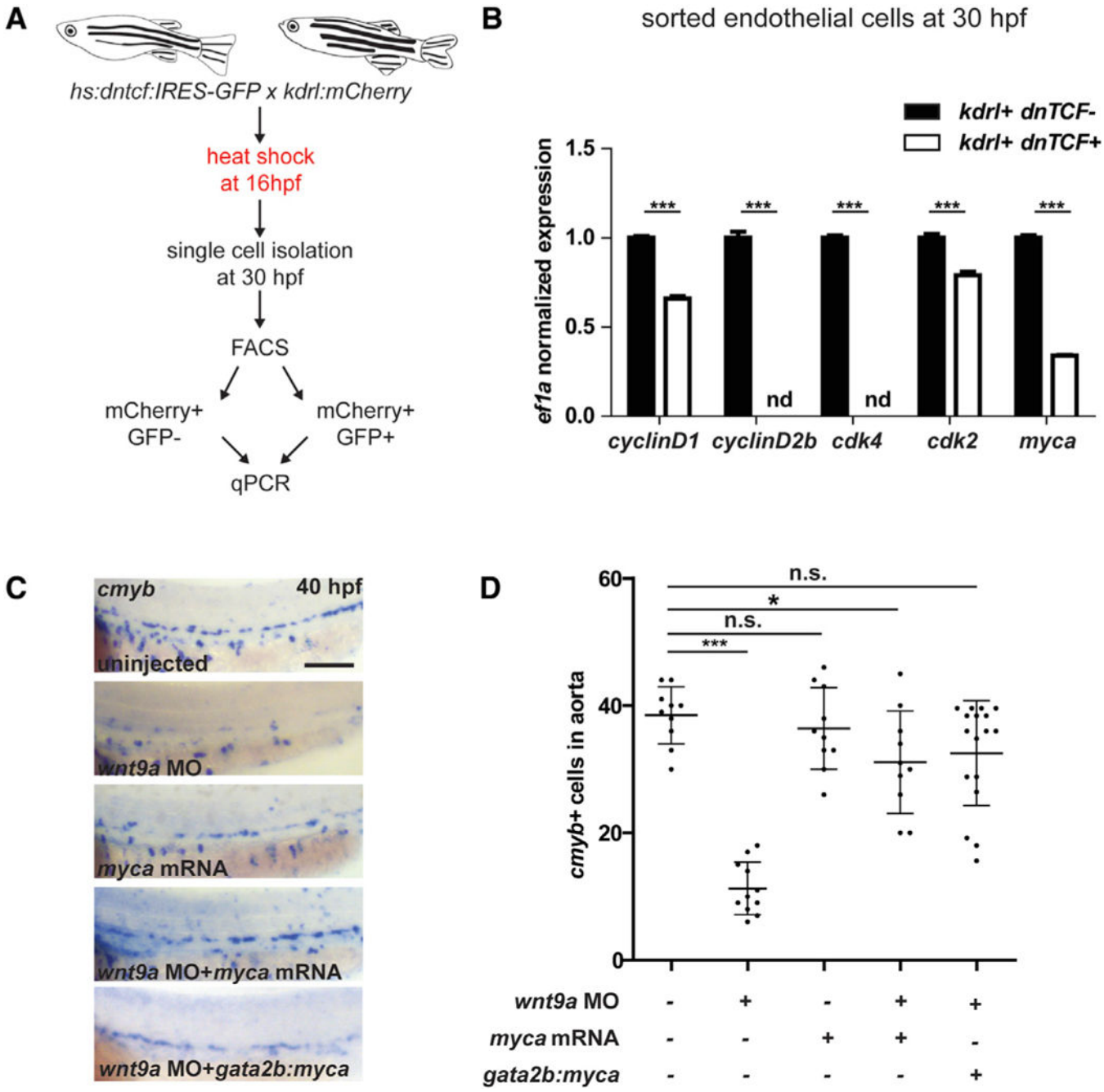

Figure 5. Wnt9a Affects Endothelial gata2b+ G1-S Cell Cycle Progression through myca

(A) Endothelial cells (mCherry+) were collected by FACS from *hs:dntcf:IRES-GFP*; *kdrl:mCherry* fish at 30 hpf after heat shock at 16 hpf (n = 100 embryos per condition).

(B) Wnt inhibited (*dntcf*;GFP+) cells were com-pared to control (GFP) cells by qPCR after being sorted by FACS.

(C and D) AB* fish were injected with wnt9a MO, *myca* mRNA, or both; *phldb4:Gal4* fish were injected with *wnt9a* MO, *UAS;myca* plasmid, and *transposase* mRNA, fixed at 40 hpf, analyzed by WISH for *cmyb* (C), and quantified in (D).

Scale bar, 100 μm. *p < 0.05; ***p < 0.001; n.s., not significant. Error bars represent SD. See also Figure S5.

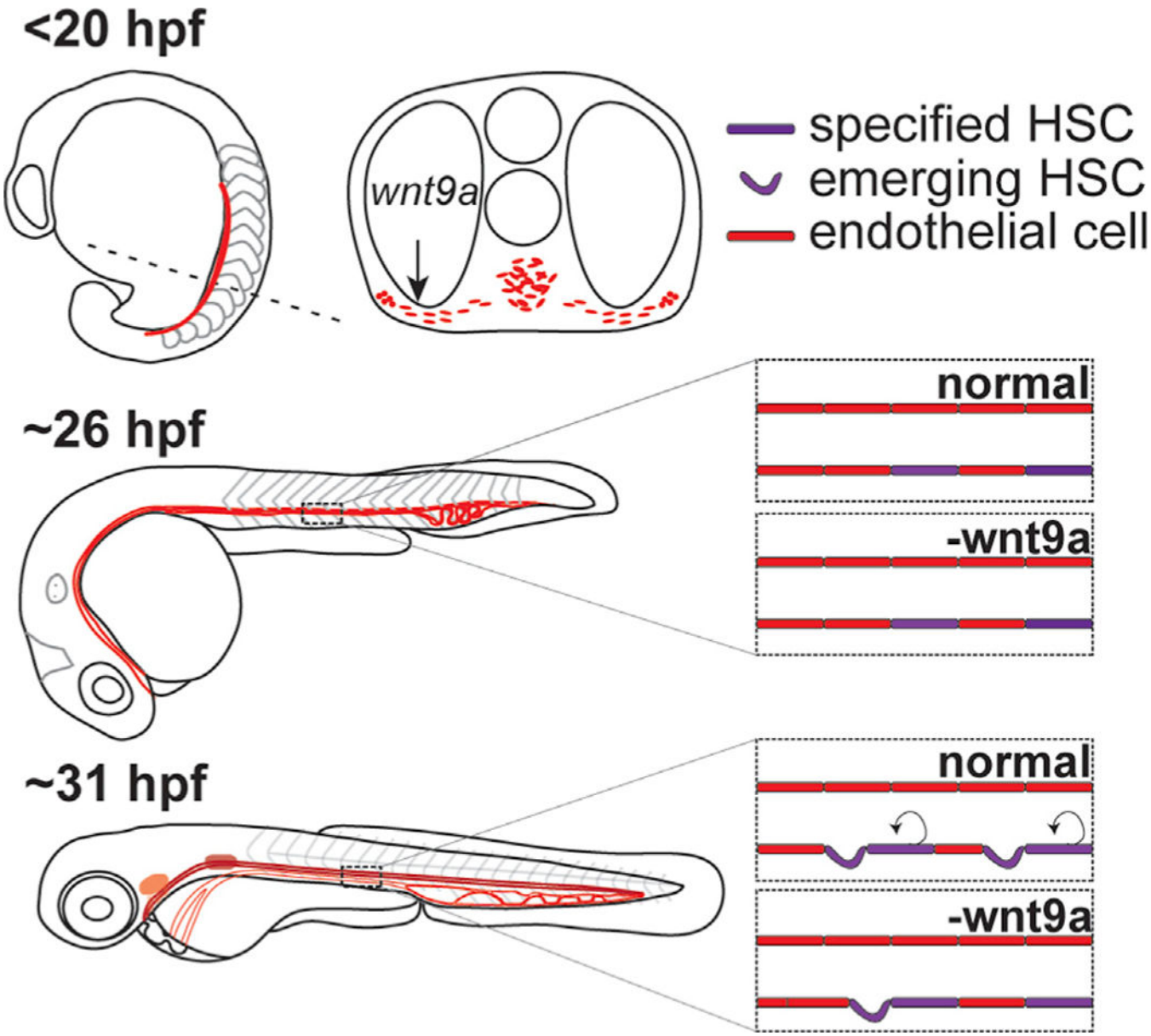

Figure 6. Early Wnt9a Cue Is Required for Later Hematopoietic Stem Cell Amplification

Prior to 20 hpf, ingressing cells of the posterior lateral mesoderm travel beneath the somites. Inductive cues direct from the somite instruct the fate of these cells, some of which are destined to become HE, and later, HSPCs. Wnt9a is expressed in the somite at this stage. By 26 hpf, the aorta has formed and HSPCs have begun to emerge. In the absence of Wnt9a, this early emergence is unaffected, indicating the HSPC fate specification has occurred properly. By 31 hpf, normal HSPCs undergo an expansion event, whereas those in Wnt9a-deficient animals do not.